Journal of Medical Virology 49:242–247 (1996)

Clinical Implications of Viral Quasispecies Heterogeneity in Chronic Hepatitis C

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To determine the clinical significance of viral quasispecies heterogeneity, 59 patients with chronic hepatitis C were studied using single-stranded conformational polymorphism (SSCP) analysis of the HCV E2 hypervariable region 1 (HVR1); of these, 48 were subsequently treated with interferon-α. The SSCP method was validated using clones of known nucleotide sequence. HVR1 was amplified in 54 of 59 (92%) patients. The median number of SSCP bands per sample was 6 (range: 2–12). Increased quasispecies heterogeneity correlated with the estimated duration of HCV infection ($P < 0.05$), parenteral-acquired HCV infection (vs. sporadic, $P < 0.05$), serum HCV RNA levels ($P < 0.05$), and HCV genotype 1 infection ($P < 0.05$), but not with age, serum AST, ALT, or Knodell score. Patients who had complete and sustained response to interferon-α (n = 11) had lower pre-treatment quasispecies heterogeneity compared to patients who had complete response with relapse (n = 18, $P < 0.05$) or no complete response (n = 16, $P < 0.01$). However, multivariate analysis revealed that HCV viremia was a stronger predictor of response to interferon-α. These findings indicate that the estimated duration of HCV carriage, serum HCV RNA levels, and HCV type 1 are important determinants for the evolution of HCV quasispecies heterogeneity; and that increased HCV quasispecies heterogeneity is another marker associated with a poor subsequent response to interferon-α.

KEY WORDS: HCV, viremia, quasispecies, heterogeneity, interferon

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded, positive sense RNA virus that is believed to replicate via a viral encoded RNA-dependent RNA-polymerase. This replication strategy has limited fidelity. Thus, HCV exists as a highly heterogenous population of closely related genomes, which are called quasispecies [Martell et al., 1992; Higashi et al., 1993; Weiner et al., 1991]. Viral quasispecies have been shown to have important pathobiological implications, including vaccine failure [Kew et al., 1984], establishment of persistent infection [Parry et al., 1990], resistance to antiviral therapy [Remington et al., 1991], and changes in virulence [Evans et al., 1985].

Determination of quasispecies heterogeneity by conventional cloning and sequencing techniques is labor intensive and expensive. This has precluded analysis of large numbers of clinical samples and has prevented extensive clinical studies. Accordingly, the clinical implications of viral quasispecies in chronic hepatitis C are unknown. Thus, the aim of the present study was to develop a rapid and reliable methods for the determination of viral quasispecies in chronic hepatitis C based on single stranded conformational polymorphism (SSCP), and to determine the clinical implications of HCV quasispecies using this established method.

MATERIALS AND METHODS

Three technical requirements for the development of a SSCP assay were anticipated. First, to assure sensitivity and avoid quasispecies selection bias, PCR primers designed from relatively well-conserved regions of the HCV E2 gene were used. However, to allow for assessment of heterogeneity, the primers flanked a hypervariable region (HVR1) [Kato et al., 1992]. Second, the 'nested' amplicons were around 200 bp long, the optimum size for SSCP since it allows easy discrimination of bands after gel separation [Hayashi, 1992]. Third, a predetermined quantity of cDNA was gel-loaded for SSCP analysis. This is of particular importance since patients with high level HCV viremia would have more amplicon per

Accepted for publication March 4, 1996.

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PCR reaction volume, and would be more likely to have detectable quasispecies. Thus, the quantity, rather than the volume, of amplicon DNA was controlled in the SSCP process.

All serum samples were centrifuged immediately after clot formation and stored at −20°C within 4 hours of venesection, conditions previously shown to optimally preserve HCV RNA [Davis et al., 1994]. HCV RNA was extracted with guanidinium thiocyanate and phenol-chloroform-isoamyl alcohol from 100 μl of serum [Davis et al., 1994]. The extracted RNA was ethanol precipitated with glycogen as a carrier and redissolved in 15 μl of sterile PCR buffer [10 mM Tris (pH 8.3 at 25°C), 50 mM KCl] containing 0.5% Nonidet P-40 and bromophenol blue (BPB, 20 mg/L) to which RNase inhibitor (8 U, Promega, Madison, WI) and dithiothreitol (DTT, 5 mM) were freshly added. In order to avoid cross-contamination, a single tube assay for both reverse transcription and the first round of PCR was used. Briefly, 4 μl of the prepared RNA solution was mixed with 16 μl of PCR buffer containing 250 μM of each of 4 dNTPs, 4.0 mM $MgCl_2$, BPB (20 mg/L), 5 mM DTT, RNase inhibitor (10 U), 16 pmol each of primers JL 128 [nucleotide (nt) 1282-1296, according to Okamoto's numbering system [Okamoto et al., 1991] 5′-TGGGATATGATG-ATGAACTGGTC-3′, sense] and JL127 [nt 1849-1820: 5′-AATGAATTCTACAACAGGGCT{TG}GG{AG}GTG-AA-3′ where { } indicate degenerate nucleotides, anti-sense), Moloney murine leukemia virus reverse transcriptase (5 U, Promega), and Taq DNA polymerase (1 U, Boehringer-Mannheim, Indianapolis, IN). After adding 40 μl of mineral oil, the tube was briefly centrifuged and placed in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, NJ) at 37°C for 45 minutes (for reverse transcription) followed by 94°C for 4 minutes, and 35 cycles each of 94°C for 1 minute (denaturing), 50°C for 1.5 minutes (annealing), and 72°C for 2 minutes (extension). Using degenerate primers for 'nested' PCR would necessarily introduce nucleotide varability within the amplified product, which would interfere with SSCP analysis. Thus, to increase the detection rate without such primer-induced nucleotide variability, two different sets of 'nested' primers from regions flanking the HVR1 region were used, in separate PCR reactions. Four μl of the first round PCR product was amplified with 60 pmol each of 'nested' primers JL129 (nt 1397-1416: 5′-GCCTTGCCTACTATTCCATG-3′, sense) and JL130 (nt 1573-1592: 5′-TTGATGTGCCAACTGCCATT-3′, anti-sense) or REG4 (nt 1397-1416: 5′-GCATAGCGTATTT-CTCCATG-3′, sense) and REG5 (nt 1573-1592: 5′-TTGATGTGCCAACTGCCATT-3′, anti-sense) for 35 cycles in a 50 μl PCR reaction as above, except that 1.8 mM $MgCl_2$ was used. The expected 196 bp amplicon, was visualized under ultraviolet illumination after ethidium bromide staining. To avoid cross-contamination during the PCR process, the general measures suggested by Kwok and Higuchi [1989] were adhered to except that different sets of pipettes were used for different phases of the work instead of using positive displacement pipettes. The concentration of the PCR amplicon was determined using a minifluorometer (Hoefer Scientific, San Francisco, CA).

To ensure reproducibility of results, an automated minigel electrophoresis system (Phast system, Pharmacia Biotech, Piscataway, NJ) was used for SSCP analysis. In two samples, the HCV E2 region was cloned and sequenced using the dideoxy chain termination method. During the initial SSCP experiments, HVR1 amplicons generated from these clones of known nucleotide sequence were used as internal positive controls. In the development of the assay, the following parameters were systematically studied: (1) amount of DNA loaded onto the gel per sample (10–200 ng); (2) gel density (4–20% polyacrylamide); (3) electrophoresis power (100–400 V); (4) electrophoresis temperature (4–30°C). Mixing experiments with various ratio of amplicons of known sequence were performed to determine whether multiple quasispecies in the same PCR product interfered with band resolution. Based on the results of this approach, an optimized SSCP protocol was established. Briefly, 25 ng of the amplicons were denatured in a solution containing 98% formamide, 0.05% bromphenol blue, and 2% glycerol at 94°C for 5 min, and quick-chilled on ice. The denatured samples were loaded on 12.5% polyacrylamide gels and separated at constant voltage (200 V) and temperature (25°C). The gels were developed using a sensitive silver staining kit (PhastGel, Pharmacia) as recommended by the manufacturer except that the duration for background reduction was prolonged to 8 minutes. The number of bands in each sample was enumerated under code by two independent observers with reference to standards with 5, 2, and 1 ng of DNA per band.

Using this established SSCP method, serum samples from 59 patients with chronic hepatitis C were studied (Table I); 47 of these patients have been previously characterized [Lau et al., 1993]. Forty eight patients were treated subsequently with recombinant interferon-α-2b at a dose of at least 3×10^6 units thrice weekly for 24 weeks. Response to interferon was classified biochemically as complete and sustained, complete with relapse, and no complete response as defined previously [Lau et al., 1993].

Serum HCV RNA was detected in all 59 patients by reverse transcription 'nested' polymerase chain reaction and quantitated using the bDNA assay (quantiplex™ HCV 1.0, Chiron Corporation, Emeryville, CA) [Lau et al., 1993]. Using type-specific oligonucleotides, this bDNA assay has been demonstrated to underestimate HCV RNA level for HCV types 2 and 3 [Collins et al., 1995]. Accordingly, HCV RNA levels were adjusted using the appropriate correction factors (i.e., × 3 for type 2 and × 2 for type 3) [Lau et al., 1995a]. As reported previously, HCV in these samples were typed using six different genotyping methods which were demonstrated to yield concordant results [Lau et al., 1995b]. HCV genotypes were assigned according to a nomenclature system proposed recently [Simmonds et al., 1994].

The data were analyzed using the SPSS-PC statistics program (SPSS Inc, Chicago, IL). Fisher's exact probability test, Mann-Whitney's non-parametric test, Pear-

TABLE I. Clinical Profile of the Patients Studied*

Patient characteristics	Data
No. of patients studied	59
Male/female	36/23
Median age (years) [range]	50 [30–72]
Mode of acquisition	
Transfusion	35
IV drug abuser/health care worker	13
Unknown ("sporadic")	11
Mean serum alanine aminotransferase (IU/L) [±SD]	161 ± 99
HIV/HBV seropositive	0
HCV RNA	
RT-PCR positive	59
bDNA positive	42
Median serum level (Meq/ml) [range]	1.8 [0.5–28.5]
Genotype: 1/2/3	39/11/9
No. of patients treated with interferon-α	48

*HIV = human immunodeficiency virus, HBV = hepatitis B virus, SD = standard deviation.

son's and Spearman's rank correlation coefficient, and multivariate analysis were applied as appropriate.

RESULTS

Optimal SSCP Conditions

The amount of cDNA gel-loaded for SSCP analysis was critical for optimum band resolution as smearing was gradually noted when more than 30 ng of amplicon DNA were electrophoresed. Similar suboptimal separation was seen when temperatures either below 20°C or above 30°C were used for SSCP electrophoresis. Interference between multiple bands was not demonstrated in mixing experiments with amplicons from clones of known sequence. SSCP analysis of two characterized clones consistently produced four discrete bands with various ratios. Using the aforementioned conditions, the sensitivity of the SSCP assay was consistently determined to be 1 ng/band (Fig. 1). Thus, viral quasispecies representing at least 4% of the total HCV population were detected.

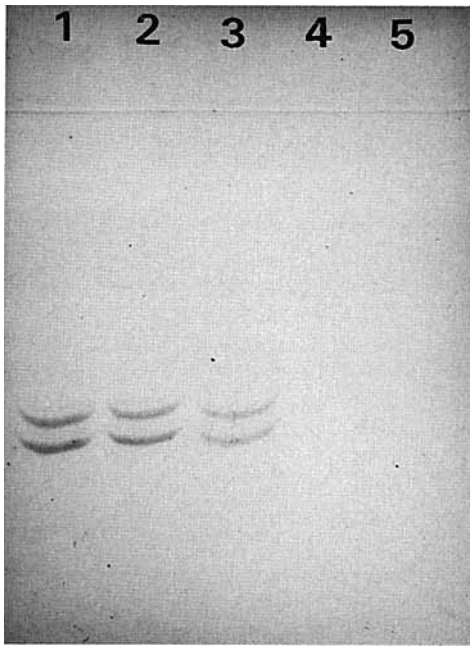

Fig. 1. Detection sensitivity of SSCP analysis using optimal electrophoretic conditions. Amount of amplicon loaded: **Lane 1:** 10 ng DNA, **lane 2:** 5 ng DNA, **lane 3:** 2 ng DNA, **lane 4:** 1 ng DNA, **lane 5:** No DNA loaded. Note that double strand amplicons appear as two discrete bands each representing single strand DNA with different conformation.

Patient Samples

The HVR1 was amplified in 54/59 (92%) patients. The median number of SSCP bands per sample was 6 (range: 2–12). The number of quasispecies correlated with the estimated duration of HCV infection ($r = 0.3$, $P < 0.05$, Fig. 2A). Patients who acquired HCV by blood product transfusion also had a greater number of quasispecies bands than those with sporadic hepatitis C ($P < 0.05$, Fig. 2B). There was also a correlation between HCV quasispecies heterogeneity and viremia ($r = 0.3$, $P < 0.05$, Fig. 2C). Infection with HCV type 1 was also associated with increased number of quasispecies ($P < 0.05$, Fig. 2D). There was no correlation between the heterogeneity of HCV quasispecies and age, serum AST, ALT, and Knodell score.

The HVR1 was detected in 45/48 patients who completed interferon-α therapy. The 11 patients who subsequently had complete and sustained response to interferon treatment had less HCV heterogeneity than either the 18 complete responders who relapsed shortly after discontinuation of therapy ($P < 0.05$) or the 16 nonresponders ($P < 0.01$; Figs. 3 and 4). However, multivariate analysis revealed that HCV viremia was a stronger predictor of response to interferon-α.

DISCUSSION

Two important points were demonstrated in this study using a sensitive and reliable SSCP method to assess HCV heterogeneity. First, the estimated duration of HCV carriage, HCV RNA levels, and infection with HCV type 1 are important determinants for the evolution of HCV quasispecies. Second, increased HCV heterogeneity is associated with a poor subsequent response to interferon-α.

The correlation between genetic heterogeneity and estimated duration of HCV carriage and serum viral RNA levels demonstrated in this study supports the concept that viral quasispecies arise as a consequence of the limited fidelity of HCV replication. On a theoretical basis, when a patient becomes infected with HCV the most replicative efficient quasispecies will predominate. Over a period of time, the lack of proofreading activity of viral RNA polymerase will lead to nucleotide variability and hence, increased genomic heterogeneity, i.e., quasispecies. The lack of an in vitro HCV culture system precludes further analysis as to whether viral quasispecies represent defective interfering particles or genuine infective virions.

Although our data demonstrates the importance of

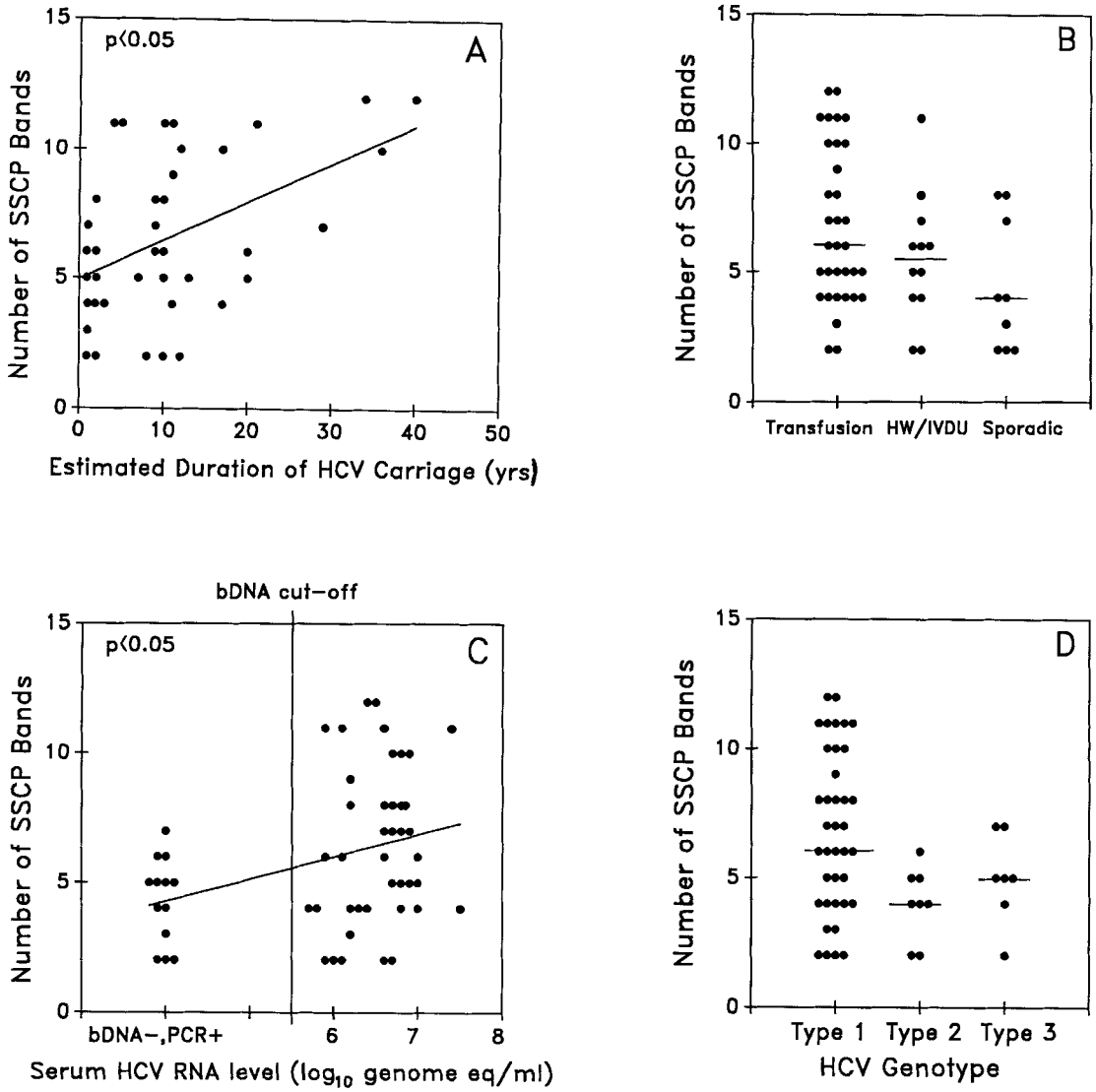

Fig. 2. Correlation between HCV quasispecies heterogeneity and estimated duration of HCV carriage **(A)**, mode of viral acquisition **(B)**, serum HCV RNA level **(C)**, and HCV genotype **(D)**.

viral factors in the evolution of HCV quasispecies, several recent reports have suggested that the host immune response may also play a critical role. Kumar et al. [1994] showed that three HCV clones generated from a patient with hypogammaglobulinemia had identical nucleotide sequences, which suggested that anti-HCV antibodies may exert selection pressure on the development of HCV quasispecies. Weiner et al. [1992] also demonstrated specific antibody binding epitopes within the HVR1 region in three patients with chronic hepatitis C. Interestingly, the appearance of a specific variant HVR1 domain was associated with recurrence of a hepatitic flare in one of the patients studied which suggested that HCV heterogeneity may be caused by immune selection. The emergence of HCV "escape mutant" populations were also suggested by Yamagushi et al. [1994] who demonstrated an increased substitution rate during acute HCV infection in three patients who became chronically infected with HCV.

There are several possible explanations for the greater viral heterogeneity shown in patients infected with HCV type 1. First, it may be related to higher serum viral levels seen in patients infected with this genotype [Hayashi et al., 1995]. However, it has been recently demonstrated that using appropriate conversion factors for the bDNA assay, serum viral levels are similar amongst HCV genotypes [Smith et al., 1996; Lau et al., 1996]. Second, RNA-dependent RNA-polymerase of HCV type 1 may be more error-prone during viral replication. Third, HCV type 1 may be more immunogenic, resulting in stronger immune selection pressure leading to increased viral heterogeneity. The recent demonstration that HCV type 2 was associated with more active liver disease in American patients did not support this notion [Lau et al.,

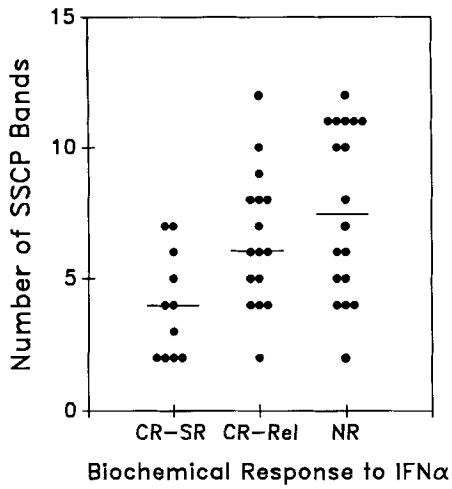

Fig. 3. Relationship between HCV quasispecies heterogeneity and response to interferon-α therapy.

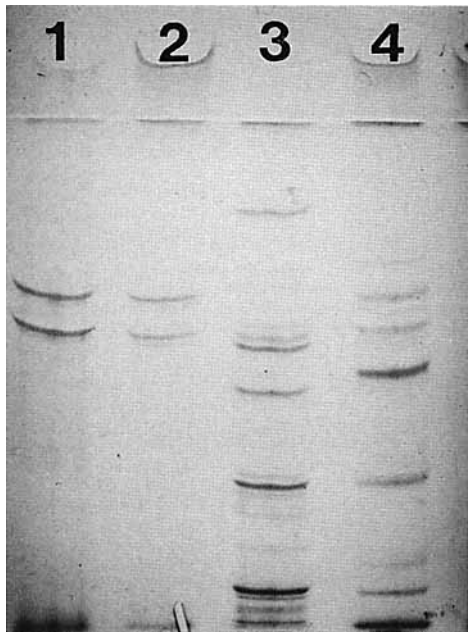

Fig. 4. SSCP analysis of four patients before interferon-α therapy; **lanes 1** and **2** are from patients who had a subsequent complete and sustained response, whereas **lanes 3** and **4** are from patients who had complete response with relapse or no response to therapy, respectively.

1995b]. The development of an in-vitro HCV culture system will help clarify these issues.

A more interesting finding of our study was the demonstration that patients with greater HCV heterogeneity, i.e., more quasispecies, had a poorer response to interferon-α therapy. Similar results were also reported, based on smaller number of patients using conventional cloning and sequencing techniques [Okada et al., 1992; Kanasawa et al., 1994], and using radioisotopic SSCP analysis [Moribe et al., 1995]. These observatios support the existence of interferon-α resistant HCV quasispecies populations or HCV quasispecies with different sensitivities to interferon-α. Although the precise mechanism is unknown, the poorer response to interferon-α may be related to either specific interferon-α resistant elements within the HCV genome [Enomoto et al., 1995], or interference by HCV defective particles that emerge with long-term viral infection [Higashi et al., 1993].

ACKNOWLEDGMENTS

The oligonucleotide primers used were synthesized by the DNA Synthesis Core Facilities, ICBR, University of Florida. The authors would like to thank Weizhen Liu for her excellent technical assistance. This study was supported in part by grants from the Children's Miracle Network Research Fund (to R.P.G.-P.), the Division of Sponsored Research of the University of Florida (DSR-RDA 1-15, 92-93), the Glaxo Institute of Digestive Health Clinical Investigator Award and the American Liver Foundation Hans Popper Liver Scholar Award (to J.Y.N.L.), and the National Institutes of Health (Clinical Research Center Grant 5M01RR0082 to the University of Florida).

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